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Dephosphorylation of microtubule-associated protein tau by protein phosphatase-1 and -2C and its implication in Alzheimer disease

Cheng-Xin Gong[a], Inge Grundke-Iqbal[a], Zahi Damuni[b], Khalid Iqbal[a,*]

[a]*New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York, NY 10314, USA*
[b]*Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, P.O. Box 850, Hershey, PA 17033, USA*

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Abstract

Microtubule-associated protein tau is abnormally hyperphosphorylated and forms the major protein subunit of paired helical filaments (PHF) in Alzheimer disease brains. The abnormally phosphorylated sites Ser-199, Ser-202, Ser-396 and Ser-404 but not Ser-46 and Ser-235 of Alzheimer tau were found to be dephosphorylated by protein phosphatase-1 and this dephosphorylation was activated by Mn^{2+}. In contrast, protein phosphatase-2C did not dephosphorylate any of these sites. Both protein phosphatase-1 and -2C had high activities towards [^{32}P]tau phosphorylated by cAMP-dependent protein kinase. These results suggest that both protein phosphatase-1 and -2C might be associated with normal phosphorylation state of tau, but only the former and not the latter phosphatase is involved in its abnormal phosphorylation in Alzheimer disease.

Key words: Tau; Protein phosphatase; Dephosphorylation; Alzheimer disease

1. Introduction

Microtubule-associated protein tau is a family of polypeptides of apparent molecular weight 50,000 to 64,000 Da that are the products of alternate splicing of a single gene [1,2]. Tau promotes the assembly of tubulin into microtubules and stabilizes their structure. Tau is a phosphoprotein and phosphorylation regulates its microtubule assembly promoting activity [3]. Recently, more research interest has been focused on tau phosphorylation because abnormally hyperphosphorylated tau has been proven to be the main protein component of paired helical filaments (PHF) which is one of the most characteristic cellular and molecular changes in Alzheimer disease (AD) brain [4]. PHF-tau and in vitro phosphorylated tau have lower activity to stimulate microtubule assembly as compared to normal tau, while dephosphorylation of PHF-tau by alkaline phosphatase can recover this activity [5,6].

Tau normally contains 2–3 mol of phosphate per mol of the protein, whereas it contains 5–9 mol of phosphate per mole of the protein in AD brain [7–9]. So far nine abnormal phosphorylation sites of PHF-tau have been identified which are not phosphorylated in normal adult brain. They are Ser-46 [10], Thr-123 [11], Ser-199 [4,12,13], Ser-202 [4,12,13], Thr-231 [14], Ser-235 [14,15], Ser-262 [14], Ser-396 [15,16], and Ser-404 [17] as numbered according to the largest isoform of human tau, tau_{441} [2]. It seems there is a disfunction of tau phosphorylation/dephosphorylation system which leads to the abnormal hyperphosphorylation of tau in AD brain. Therefore, it is of great interest to identify the protein kinases and protein phosphatases which are involved in the reversible process of tau phosphorylation.

Phosphoseryl and phosphothreonyl protein phosphatases (PP) have been classified by Cohen and Ingebritsen into four main types, i.e. PP-1, PP-2A, PP-2B and PP-2C [18]. These protein phosphatases are present in significant concentrations in human brain [19]. PP-2A and PP-2B have been shown to dephosphorylate tau phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase and cAMP-dependent protein kinase (PKA) [20,21]. Recently, we found that PP-2A (Gong et al., manuscript submitted for publication) and PP-2B [22] also dephosphorylate Alzheimer tau at the abnormal phosphorylation sites. In this study, we describe the activities of the other two types of protein phosphatases, PP-1 and PP- 2C, towards tau phosphorylated by PKA and Alzheimer disease abnormally phosphorylated tau (AD P-tau).

* Corresponding author. Fax: (1) (718) 494 1080.

Abbreviations: AD, Alzheimer disease; AD P-tau, Alzheimer disease abnormally phosphorylated tau; PHF, paired helical filaments; PKA, cAMP-dependent protein kinase; PP, protein phosphatase.

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2. Materials and methods

2.1. Materials

Phosphorylase kinase was purified from rabbit skeletal muscle by the method of Cohen [23]. PKA was purchased from Sigma, St. Louis, MO, USA. Rabbit skeletal muscle PP-1 was purchased from Upstate Biotechnology Inc., Lake Placid, NY. PP-2B (holoenzyme) was purified from bovine brain according to the method of Sharma et al. [24]. PP-2C was purified from bovine kidney as described previously [25]. Polyclonal antibodies 102c were raised as previously reported [10]. Monoclonal antibodies Tau-1 and PHF-1 were kindly provided by Drs. L.I. Binder [26] and S. Greenberg [27], respectively; SMI33, SMI31, goat anti-mouse IgG and peroxidase–anti-peroxidase complex were purchased from Sternberger Monoclonals Inc., Baltimore, MD. Alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Bio-Rad, Hercules, CA.

2.2. Isolation of tau

AD P-tau and normal human tau were isolated from autopsied brains of a 70-year-old male with Alzheimer disease and a 51-year-old male normal case, respectively [9]. Briefly, AD P-tau was isolated from a non-neurofibrillary tangle pool, the 27,000 × *g* to 200,000 × *g* fraction of the Alzheimer brain homogenate by extraction in 8 M urea, followed by dialysis against Tris buffer. This AD P-tau enriched fraction is readily soluble in buffer and abnormally phosphorylated as PHF-tau [9]. Normal human tau was purified from the 35–45% ammonium sulfate precipitates of 200,000 × *g* brain supernatant, followed by acid treatment (pH 2.7) and chromatography on a phosphocellulose column (Cellulose Phosphate P11, Whatman).

2.3. Preparation of [^{32}P]phosphorylase kinase and determination of protein phosphatase activities

[^{32}P]Phosphorylase kinase (1.9 mol ^{32}P incorporated/335,000 × *g*) phosphorylated by PKA was prepared as reported previously [19]. The activities of PP-1, PP-2B and PP-2C were measured by counting the radioactivity released from [^{32}P]substrate as previously described [19]. The reaction mixtures contained 50 mM Tris, pH 7.0, 20 mM β-mercaptoethanol, 1.0 mM $MnCl_2$ and 1.0 μM [^{32}P]phosphorylase kinase for PP-1. For PP-2B and PP-2C activities, $MnCl_2$ was substituted by 1.0 mM $CaCl_2$ and 1.0 μM calmodulin, and 10 mM $MgCl_2$, respectively. One unit of protein phosphatase activity is defined as that amount which catalyzes the release of 1.0 nmol phosphate per min from [^{32}P]phosphorylase kinase at 30°C.

2.4. Treatment of AD P-tau with protein phosphatases

Unless otherwise stated, dephosphorylation of AD P-tau was carried out at 30°C in 50 mM Tris, pH 7.0, 10 mM β-mercaptoethanol, 0.1 mg/ml BSA, 50 μg/ml AD P-tau and PP-1, PP-2B or PP-2C. In some experiments, several effectors were added in the reaction mixture (see section 3). The reaction was started by addition of the enzyme. After appropriate incubation times (see figure legends), reactions were stopped by addition of 5 volumes of cold acetone to precipitate proteins. The precipitated protein samples were dissolved in SDS-PAGE sample buffer and heated at 95°C for 4 min, followed by 10% SDS-PAGE. Immunoblotting was carried out as described previously [4]. The primary antibodies for immunoblotting and their epitopes have all been previously characterized. They are phosphorylation-dependent as well as site-specific. Briefly, antibodies 102c [10], Tau-1 [4,12,13] and SMI33 [15] recognize dephosphorylated form of tau at sites Ser-46, Ser-199/Ser-202 and Ser-235, respectively. Antibodies SMI31 [15] and PHF-1 [28] recognize tau phosphorylated at Ser-396/Ser-404 and Ser-396, respectively. These antibodies were used at dilutions of 0.4 μg/ml for 102c, 1:100 for SMI31, 1:500 for SMI33 and PHF-1, and 1:500,000 for Tau-1.

2.5. Preparation of [^{32}P]tau and dephosphorylation of [^{32}P]tau by protein phosphatases

Tau purified from normal human brain was phosphorylated with [^{32}P]ATP by PKA as described by Scott et al. [29]. About 2 mol ^{32}P/mol tau was incorporated by PKA. Dephosphorylation of [^{32}P]tau by PP-1, PP-2B and PP-2C was carried out employing the same conditions as those described above for the dephosphorylation of AD P-tau. The phosphatase activities were measured by counting the radioactivity released from [^{32}P]tau as previously described [19].

3. Results

3.1. Definition of PP-1 and PP-2C activities and their modulation by Mn^{2+} and Mg^{2+}

To observe and compare the potential dephosphorylation of AD P-tau by various protein phosphatases, the same amount of enzyme activity of each phosphatase should be used. Hence we employed [^{32}P]phosphorylase kinase as a substrate to standardize the activities of PP-1 and PP-2C. PP-1 and PP-2C activities are modulated by cations and each enzyme preparation responds differently to divalent cations [30]. We therefore determined PP-1 and PP-2C activities in the absence and presence of either Mn^{2+} or Mg^{2+} using [^{32}P]phosphorylase kinase as a substrate. As shown in Fig. 1, PP-1 was activated by 1.0 mM Mn^{2+} but inhibited by 10 mM Mg^{2+}. PP-2C was Mg^{2+}-or Mn^{2+}-dependent, and no activity was detected in the absence of Mg^{2+} or Mn^{2+}. Highest activities were obtained using 1.0 mM Mn^{2+} for PP-1 and 10 mM Mg^{2+} for PP-2C. Hence these conditions were used to study the in vitro dephosphorylation of AD P-tau and PKA-phosphorylated tau.

3.2. Treatment of AD P-tau with PP-1 and PP-2C

We have previously found that PP-2A dephosphorylated abnormal phosphorylation sites Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau (Gong et al., manuscript submitted for publication), and

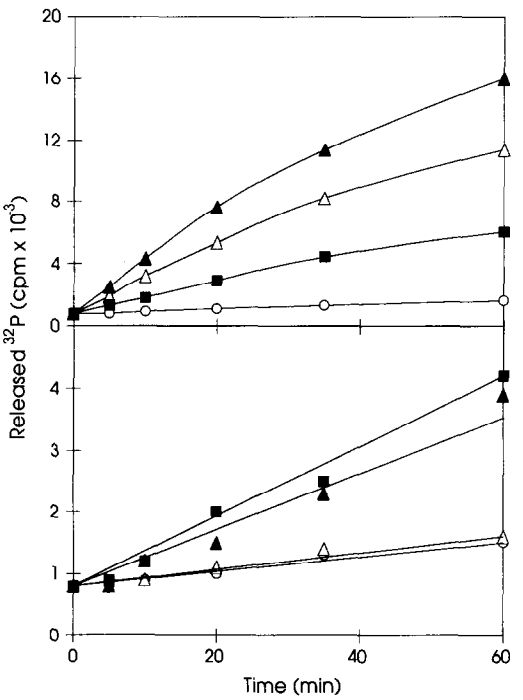

Fig. 1. PP-1 (upper panel) and PP-2C (lower panel) activities in the presence or absence of various divalent metal ions. Dephosphorylation reactions were carried out using [^{32}P]phosphorylase kinase as substrate as described in section 2, and in the presence of 1.0 mM EDTA (△), 1.0 mM $MnCl_2$ (▲) or 10 mM $MgCl_2$ (■). The open circles (○) indicate assays in the absence of the protein phosphatases.

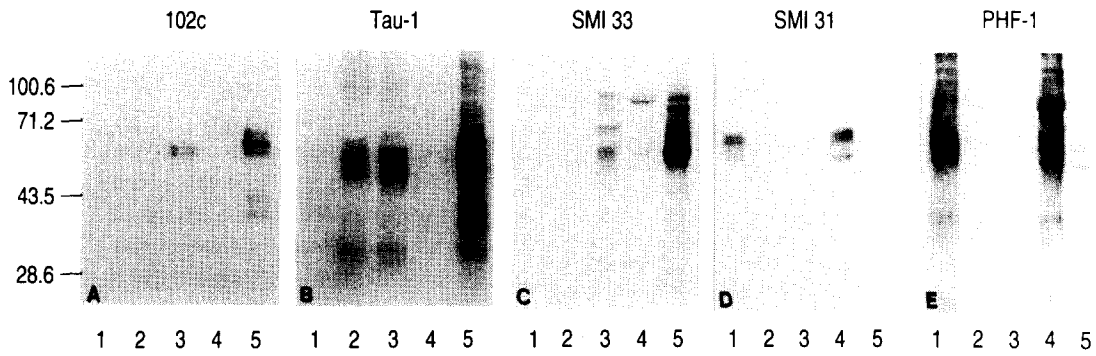

Fig. 2. Dephosphorylation of AD P-tau by PP-1, PP-2B and PP-2C. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 2.0 units/ml PP-1 (lane 2), PP-2B (lane 3) or PP-2C (lane 4) at 30°C for 60 min as described in Materials and Methods; lane 5 shows untreated normal human tau for comparison. Reaction mixtures for PP-1 and PP-2C also contained 1.0 mM $MnCl_2$ and 10 mM $MgCl_2$, respectively. For PP-2B, 1.0 μM calmodulin, 1.0 mM $CaCl_2$ and 1.0 mM $MnCl_2$ were included. Five phosphorylation-dependent antibodies were used for immunoblotting as shown above each panel to monitor dephosphorylation of the specific sites of AD P-tau. 102c (A), Tau-1 (B) and SMI33 (C) recognize dephosphorylated forms, whereas SMI31 (D) and PHF-1 (E) recognize phosphorylated forms of tau at specific sites as described in section 2. Molecular weight (kDa) markers are indicated at left margin of the figure. SMI33, a monoclonal antibody to neurofilament heavy/medium subunits, crossreacts with tau unphosphorylated at Ser-235 [15]. In lane 4, panel C, the 88 kDa protein band stained prominently without a corresponding staining in the tau region is probably not tau.

that in addition to above sites, PP-2B also dephosphorylated another abnormal phosphorylation site, Ser-235 [22]. In the present study, using immunoblots with five site-specific phosphorylation-dependent antibodies which recognize six abnormal phosphorylation sites of AD P-tau, we have further examined whether PP-1 and PP-2C can also dephosphorylate these abnormal phosphorylation sites at optimum in vitro conditions. PP-2B was employed as a positive control. We found that PP-1 unmasked the epitope of antibody Tau-1 and blocked the epitopes of antibodies SMI31 and PHF-1, but failed to unblock the epitopes of antibodies 102c and SMI33 (Fig. 2). PP-2C did not change any of these epitopes. These results indicate that PP-1 dephosphorylates abnormal phosphorylation sites Ser-199, Ser-202, Ser-396 and Ser-404 but not Ser-46 and Ser-235 of AD P-tau. Whereas PP-2C had no effect on dephosphorylation of above sites.

The rate of dephosphorylation of Ser-199/Ser-202, Ser-396/Ser-404 and Ser-396 of AD P-tau by PP-1 was determined using immunoblots with Tau-1, SMI31 and PHF-1, respectively. The time course showed a rapid change of epitopes of AD P-tau towards these three antibodies (Fig. 3). Within 20–30 min incubation of AD P-tau with PP-1, staining of Tau-1 became maximal and those of both PHF-1 and SMI31 disappeared completely. These results suggested that the four phosphorylation sites of AD P-tau can be easily hydrolyzed by PP-1.

We have also investigated the dephosphorylation of AD P-tau at various conditions (Fig. 4). In the absence of metal (1.0 mM EDTA present), PP-1 could also dephosphorylate AD P-tau at Ser-396 but the activity was low. Dephosphorylation of AD P-tau by PP-1 was strongly activated by 1.0 mM Mn^{2+} but inhibited by 10 mM Mg^{2+}. We further investigated the required concentration of Mn^{2+} for this activation. The activation was observed at 10 μM Mn^{2+} and it reached maximum at about 100 μM Mn^{2+} (Fig. 5); the physiological level of Mn^{2+} is 5–11 μM in brain [33].

3.3. *Dephosphorylation of [^{32}P]tau by PP-1 and PP-2C*

Tau protein is known to be phosphorylated in vitro at Ser-214, Ser-324, Ser-356, Ser-409 and Ser-416 by PKA [29]. So far none of these sites have been reported to be abnormally phosphorylated in Alzheimer disease brain, but they may be involved in normal phosphorylation of tau. We therefore asked whether these non-abnormal

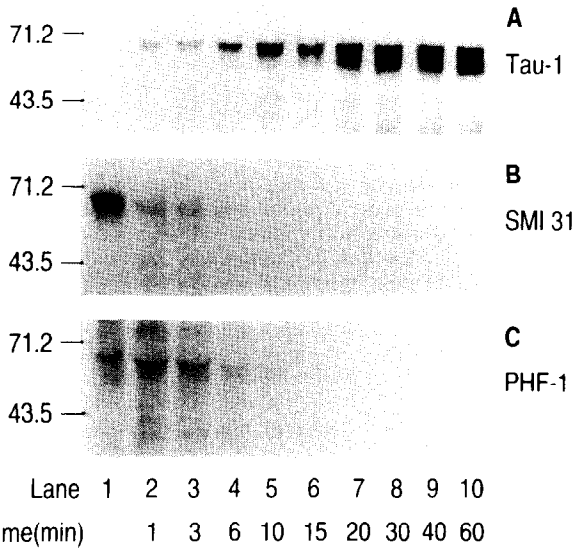

Fig. 3. Time course of dephosphorylation of AD P-tau by PP-1. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 1.0 unit/ml PP-1 as described in Fig. 2 at 30°C for different time intervals (lanes 2–10). Phosphorylation-dependent antibodies Tau-1 (A), SMI31 (B) and PHF-1 (C) were used to monitor the dephosphorylation. Molecular weight (kDa) markers are indicated at the left of each panel.

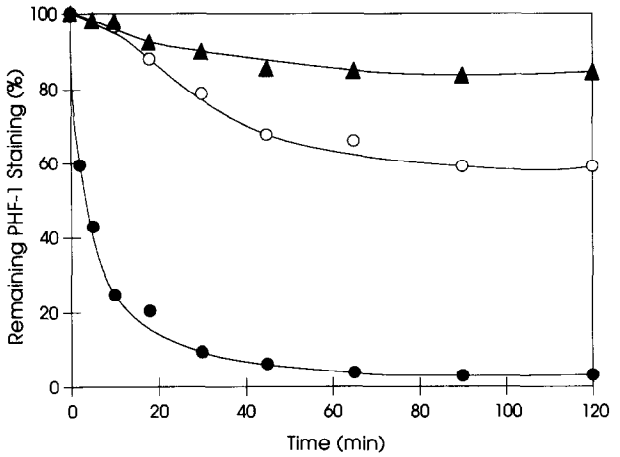

Fig. 4. Effect of Mn^{2+} and Mg^{2+} on dephosphorylation of AD P-tau by PP-1. AD P-tau was incubated with 1.0 unit/ml PP-1 in the presence of either 1.0 mM EDTA (○), 1.0 mM Mn^{2+} (●) or 10 mM Mg^{2+} (▲) at 30°C for different time intervals as described in section 2. After incubation, AD P-tau was subjected to immunoblotting with monoclonal antibody PHF-1 which stains only phosphorylated forms of tau, followed by densitometric scanning. Dephosphorylation is expressed by percentage of remaining PHF-1 staining.

phosphorylation sites of tau can be dephosphorylated by either PP-1 and PP-2C. Interestingly, even though PP-1, PP-2B and PP-2C had obviously different effects on dephosphorylation of abnormal phosphorylation sites of AD P-tau, they had similar high activities towards [^{32}P]tau phosphorylated by PKA (Fig. 6).

4. Discussion

In order to examine whether there is a defect of protein phosphatase(s) that might be involved in the abnormal phosphorylation of tau in AD brain, it is essential to identify which protein phosphatase(s) can dephosphorylate the AD P-tau. We have recently found that PP-2A and PP-2B rapidly dephosphorylate AD P-tau in vitro ([22] and Gong et al. manuscript submitted for publication). The present study shows that PP-1 also dephosphorylates AD P-tau at some of the sites whereas PP-2C has no activity towards any of the sites studied. Although three of four types of protein phosphatases can dephosphorylate AD P-tau, they have different site specificities. Six of nine known abnormal phosphorylation sites of AD P-tau have been examined in these studies. They are Ser-46, Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404. All of them can be dephosphorylated by PP-2B; PP-2A can dephosphorylate all except S-235; and PP-1 dephosphorylates Ser-199, Ser-202, Ser-396 and Ser-404 but neither Ser-46 nor Ser-235. Hence at least four abnormal phosphorylation sites, Ser-199, Ser-202, Ser-396 and Ser-404, can be dephosphorylated by the three enzymes, PP-1, PP-2A and PP-2B. These results indicate that the regulation of phosphorylation level of tau is very complex and more than one protein phosphatase might be involved in hyperphosphorylation of tau in AD.

When [^{32}P]phosphorylase kinase was used as a substrate to determine protein phosphatase activities, PP-1 activity was about 20-fold higher than PP-2C activity in human brain extracts [19]. In the present study, also using [^{32}P]phosphorylase kinase as a substrate to define the activities of both PP-1 and PP-2C, 1.0 unit/ml of PP-1 almost completely dephosphorylated Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau in 20–30 min, but 2.0 units/ml of PP-2C did not dephosphorylate AD P-tau at any sites studied at the optimal in vitro conditions. Taken together, these results suggest that AD P-tau is not a substrate for PP-2C.

Tau isolated from adult brain normally contains 2–3 mol of phosphate per mol of the protein [7–9]. However, neither the phosphorylation sites nor the responsive kinase(s) have yet been fully elucidated. Normal tau might be partially phosphorylated at Ser-202 and Ser-404 [31,32], but to date other sites have not been excluded to be phosphorylated. PKA is known to phosphorylate tau at Ser-214, Ser-234, Ser-356, Ser-409 and Ser-416 [29]. PP-2C as well as PP-1 and PP-2B can release about 80% radioactivity from PKA-phosphorylated [^{32}P]tau in 60 min, suggesting that these phosphatases dephosphorylate most of these phosphorylation sites of tau. Therefore, even if it is not involved in abnormal phosphorylation of Alzheimer tau, PP-2C may be associated with the regulation of phosphorylation level of normal tau.

The present study also shows that PKA-phosphorylated tau and AD P-tau serve as different substrates for protein phosphatases. AD P-tau can be dephosphorylated by PP-1, PP-2A and PP-2B but not by

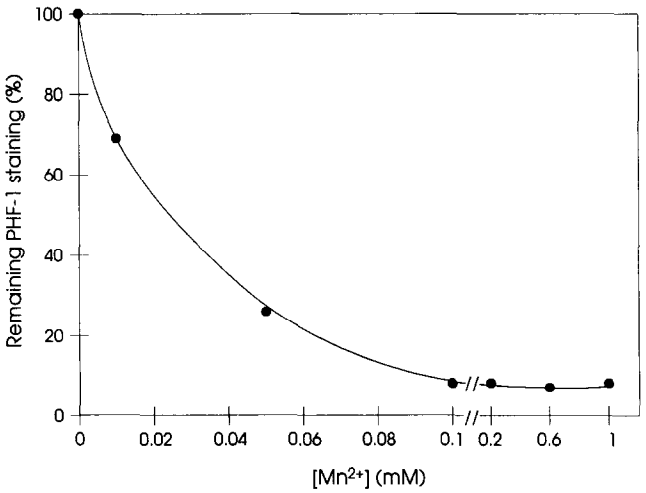

Fig. 5. Dephosphorylation of AD P-tau by PP-1 at various concentrations of Mn^{2+}. AD P-tau was incubated with 1.0 unit/ml PP-1 in the presence of various concentrations of $MnCl_2$ at 30°C for 60 min as described in section 2. After incubation, AD P-tau was subjected to immunoblotting with PHF-1 (which stains only tau phosphorylated at Ser-396) followed by densitometric scanning. Dephosphorylation is expressed by percentage of remaining PHF-1 staining.

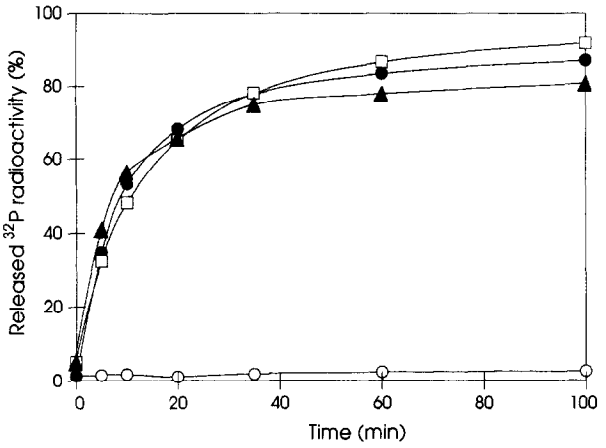

Fig. 6. Dephosphorylation of PKA-phosphorylated tau by PP-1, PP-2B and PP-2C. PKA-phosphorylated tau (0.1 mg/ml) was incubated either without (○) or with 0.4 unit/ml of PP-1 (●), PP-2B (□) or PP-2C (▲) at 30°C for different time intervals as described in section 2. The reaction mixtures also included 1.0 mM $MnCl_2$ for PP-1, 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 1.0 mM $MnCl_2$ for PP-2B, and 10 mM $MgCl_2$ for PP-2C.

PP-2C, whereas PKA-phosphorylated tau is almost an equally good substrate for PP-1, PP-2B and PP-2C. Whether the completely different behavior of AD P-tau and PKA-phosphorylated tau as a substrate for PP-2C is due to different phosphorylation sites, and/or due to different protein conformations remains to be investigated.

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